National Institute of Standards & Technology

# Certificate of Analysis

## Standard Reference Material® 1955

### Homocysteine and Folate in Frozen Human Serum

This Standard Reference Material (SRM) is intended primarily for use in evaluating the accuracy of clinical procedures for the determination of homocysteine and folate (in various forms) in human serum. It is also intended for use in validating working or secondary reference materials. A unit of SRM 1955 consists of three bottles of frozen human serum, each of different analyte concentration levels. Each bottle contains 1 mL of human serum.

**Certified Concentration Values:** The certified concentrations and their expanded uncertainties for total homocysteine (tHCY) and 5-methyltetrahydrofolic acid (5MT) are listed in Table 1. The certified concentrations of tHCY were determined at NIST using a combination of higher order reference measurement procedures based upon isotope dilution with gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS), and liquid chromatography/tandem mass spectrometry (LC/MS/MS) [1–3]. The certified concentrations for 5MT were determined by a combination of reference measurement procedures based upon isotope dilution LC/MS/MS performed at NIST [3,4] and the Centers for Disease Control and Prevention (CDC) [5]. The methods performed at NIST for tHCY and 5MT are recognized as approved higher order reference measurement procedures by the Joint Committee on Traceability in Laboratory Medicine (JCTLM) [6]. The certified concentrations apply only to serum thawed to room temperature, 20 °C to 25 °C (see "Instructions for Use").

**Reference Concentration Values:** The reference concentrations for folic acid (FA) and their expanded uncertainties are listed in Table 2. The reference concentrations were determined using LC/MS/MS-based methods at NIST and CDC. Because agreement among the methods for this low-level analyte does not meet NIST criteria for a certified value, the results are listed as reference values [7].

**Information Values:** CDC provided additional results for total folate, 5-formyltetrahydrofolic acid (5FT), tHCY, and vitamin $B_{12}$ (cobalamin). These results are listed as method-specific results in Table 3. For those analytes for which multiple methods were used, it was decided that it was more useful to the user community to list the individual results rather than combining them, since the different methods may not be measuring the same entities.

**Expiration of Certification:** The certification of this SRM is valid until **31 December 2008**, within the measurement uncertainties specified, provided the SRM is handled and stored in accordance with the instructions given in the certificate. However, the certification is nullified if the SRM is damaged, contaminated, or modified.

**Maintenance of SRM Certification:** NIST will monitor this SRM over the period of its certification. If changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet) will facilitate notification.

The overall direction and coordination of the analyses at NIST were under the chairmanship of M.J. Welch of the NIST Analytical Chemistry Division.

Stephen A. Wise, Chief
Analytical Chemistry Division

Gaithersburg, MD 20899
Certificate Issue Date: 06 October 2005

Robert L. Watters, Jr., Chief
Measurement Services Division

The analytical measurements at NIST were performed by B.C. Nelson, M.B. Satterfield, and L.T. Sniegoski of the NIST Analytical Chemistry Division. Measurements at CDC were performed by M. Zhang, Z. Fazili, S. Strider, and L. Jia, under the direction of C. Pfeiffer.

The statistical analysis of the NIST data was performed by A. Hornikova and N.F. Zhang of the NIST Statistical Engineering Division.

The support aspects involved in the issuance of this SRM were coordinated through NIST Measurement Services Division.

## NOTICE AND WARNINGS TO USERS

**SRM 1955 IS INTENDED FOR IN-VITRO DIAGNOSTIC USE ONLY. THIS IS A HUMAN SOURCE MATERIAL. HANDLE PRODUCT AS A BIOHAZARDOUS MATERIAL CAPABLE OF TRANSMITTING INFECTIOUS DISEASE**. The supplier of this serum has reported that each donor unit of serum or plasma used in the preparation of this product was tested by an FDA-approved method and was found to be nonreactive for HbsAG, HCV, and HIV-1 antibodies. However, no known test method can offer complete assurance that hepatitis B virus, hepatitis C virus, HIV, or other infectious agents are absent from this material. Accordingly, this human blood-based product should be handled at the Biosafety Level 2 or higher as recommended for any POTENTIALLY INFECTIOUS HUMAN SERUM OR BLOOD SPECIMEN in the CDC/National Institutes of Health (NIH) Manual [8].

**Storage:** The serum is shipped frozen (on dry ice), and upon receipt, should be stored frozen until ready for use. A freezer temperature of −20 °C is acceptable for storage up to one week. If a longer storage time is anticipated, the material should be stored at or below −50 °C. The SRM should not be exposed to sunlight or ultraviolet radiation. Storage of thawed material at room or refrigerator temperatures may result in changes in the analyte concentrations.

**Stability:** The material is kept at −80 °C for long term storage at NIST. NIST will continue to monitor the stability of the analytes in this material and will notify purchasers of the material of any changes in the certified concentrations.

## INSTRUCTIONS FOR USE

Bottles of the SRM to be analyzed should be removed from the freezer and allowed to stand at room temperature until thawed. After the material is thawed to room temperature, it should be used **immediately**. The material should be swirled gently to mix it before aliquots are withdrawn.

## SOURCE, PREPARATION, AND ANALYSIS[1]

**Source of Material:** SRM 1955 Homocysteine and Folate in Frozen Human Serum was prepared by Aalto Scientific Ltd. (Carlsbad, CA).

**Preparation of Material:** The material was prepared from a human serum master pool. The analyte concentrations in the level 1 and level 2 materials were unfortified. The analyte concentrations in the level 3 material were fortified. The level 2 material is equivalent to the human serum master pool. The level 1 material was prepared by diluting the level 2 material with phosphate-buffered saline solution, pH 7.04. The level 3 material was prepared by adding appropriate quantities of homocystine and 5–methyltetrahydrofolic acid to the level 2 material.

## ANALYTICAL METHODS

**Homocysteine Measurements at NIST:** Homocysteine was measured using three isotope dilution mass spectrometry approaches [1–3]. For the LC/MS and most of the LC/MS/MS measurements, three independent sets were run. A set consisted of two samples per bottle from two bottles of each of the three levels. Each sample was spiked with a known amount of the internal standard, homocysteine-$d_4$. After reduction of the sample by addition of dithiothreitol (DTT), sample clean-up was performed using anion exchange solid phase extraction. The solvent from the extraction was evaporated to < 50 μL and the residue was dissolved in 1.5 % DTT in water. A commercial LC/MS/MS instrument with an electrospray ionization source and a triple quadrupole mass analyzer was used for the analysis. The LC separations were carried out using a commercial pentafluorophenyl column (25 cm × 4.6 mm,

[1]Certain commercial equipment, instrumentation, or materials are identified in this certificate to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the NIST, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

5 μm particle diameter) with an isocratic mobile phase at a flow rate of 0.5 mL/min. Electrospray ionization in the positive mode was used with selected ion monitoring to measure the $[M + H]^+$ ions at *m/z* 136 and *m/z* 140 for homocysteine and homocysteine-$d_4$, respectively. Selected reaction monitoring of two transitions were carried out at *m/z* 136 → *m/z* 90 and *m/z* 136 → *m/z* 118 for hcy and *m/z* 140 → *m/z* 94 and *m/z* 140 → *m/z* 122 for hcy-$d_4$ on the same samples. Analyte concentrations were calculated by linear interpolation from calibration curves constructed independently for each set of samples. Three additional sets, each consisting of two aliquots from one vial of each level, were prepared for LC/MS/MS measurements following NIST 5MT method 3 [3] described below with measurements using the MS/MS conditions described above.

For measurements using GC/MS, three independent sets were run. A set consisted of two samples per bottle from two bottles of each of the three levels. Each sample was spiked with a known amount of the internal standard, homocystine-$d_8$. DDT in sodium hydroxide solution was used to break disulfide bonds and release homocysteine and homocysteine-$d_4$, which were then isolated by absorption on anion exchange resin, followed by elution, concentration, derivatization with N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA), and GC/MS with selective ion monitoring. The GC/MS measurements were performed using a mass selective detector with two different 30-meter capillary columns (one with a 5 % phenylmethylpolysiloxane stationary phase, the other with 50 % phenylmethylpolysiloxane). Two different pairs of ions (*m/z* 420/424 and 318/322) were monitored.

**Homocysteine Measurements at CDC:** CDC used two methods to measure homocysteine. These data were not used to calculate the certified values, but are presented to demonstrate the commutability of this SRM for routine methods for the determination of homocysteine. For the first method [9], total homocysteine (tHCY) was measured by isocratic liquid chromatography with fluorometric detection (LC-FD) at 385 nm excitation and 515 nm emission after reduction of protein-bound and oxidized thiols (disulfides and mixed disulfides) to free thiol with tris(2−carboxyethyl)phosphine (TCEP), protein precipitation with trichloroacetic acid, and fluorescent derivatization with ammonium 7−fluorobenzo−2−oxa−1,3−diazole−4−sulfonate (SBD-F). The separation of the obtained thiol−derivatives was performed isocratically within 7 min, with the use of an acetate mobile phase at pH 5.5. Cystamine was used as an internal standard. Quantitation was by peak area ratio (analyte to internal standard) and is based on a three-point standard curve in a serum matrix. For the second method [10], tHCY was measured by the "Abbott Homocysteine assay", a fully automated fluorescence polarization immunoassay (FPIA) from Abbott Diagnostics. In brief, DDT reduces homocysteine bound to albumin and to other small molecules, homocystine, and mixed disulfides, to free thiol. S-adenosyl-homocysteine (SAH) hydrolase catalyzes conversion of homocysteine to SAH in the presence of added adenosine. In the subsequent steps, a specific monoclonal antibody and a fluoresceinated SAH analog tracer constitute the FPIA detection system. Serum tHCY concentrations were calculated by the Abbott AxSym® using a machine-stored calibration curve.

**Folate Measurements at NIST:** For measurements of 5MT at NIST, three variations of LC/MS/MS-based methodology were used [3,4].

> **Method 1:** For each sample, a 500 μL aliquot of SRM 1955 was spiked with $[^{13}C_5]$-5MT stock standard, immediately diluted 1:1 with 10 % ascorbic acid/1 % EDTA solution and allowed to equilibrate on ice for ≥ 15 min. 5MT was extracted from samples using $C_{18}$ SPE cartridges: methanol, water and 1 % ascorbic acid/1 % EDTA solution were used for conditioning, 95/5 water/methanol solution was used for washing and 1 % ascorbic acid/1 % formic acid in 50/50 water/methanol was used for analyte elution. A commercial LC/MS/MS instrument with an electrospray ionization source and a triple quadrupole mass analyzer was used for extract analysis. LC separations were carried out using a phenylpropyl LC column (3.9 mm x 150 mm, 4 μm particle diameter). The mobile phase elution program was (flow rate of 0.35 mL/min): Eluent A = 1 % formic acid in water; Eluent B = 1 % formic acid in methanol; time program = 0 min, 70 % A/30 % B; 5.0 min, 70 % A/30 % B; 5.1 min, 0 % A/100 % B; 7 min, 0 % A/100 % B; 7.1 min, 70 % A/30 % B; 10 min, 70 % A/30 % B. LC/MS/MS detection and quantification of 5MT and $[^{13}C_5]$-5MT was conducted in multiple reaction monitoring (MRM) mode. MRM transitions for each analyte were individually optimized for protonated analyte molecules $[M + H]^+$ and stable, protonated fragments. The relevant MRM mass transitions were *m/z* 460 → *m/z* 313 for 5MT and *m/z* 465 → *m/z* 313 for $[^{13}C_5]$-5MT. Analyte concentrations were calculated by linear interpolation from calibration curves constructed independently for each set of samples.
>
> **Method 2:** Samples were spiked and equilibrated as with Method 1. Sample proteins were precipitated with metaphosphoric acid and the remaining supernatant was neutralized by the addition of 0.4 mol/L $K_2HPO_4$/0.5 mol/L NaOH. 5MT was extracted from samples using solid phase affinity extraction (SPAE) columns: 1 % ascorbic acid/0.1 mol/L glycine-HCl solution (pH 3.0), water and 50 mmol/L potassium phosphate buffer (pH 7.4) were used for conditioning, water was used for washing and 1 % ascorbic

acid/0.1 mol/L glycine-HCl solution (pH 3.0) was used for analyte elution. The LC/MS/MS was performed as in Method 1.

**Method 3:** (for 5MT and FA), sample preparation and instrumentation were as for Method 1. LC separations were carried out using a $C_{18}$ LC column (4.6 mm × 150 mm, 5 µm particle diameter). The mobile phase elution program was (flow rate = 0.35 mL/min): Eluent A = 1 % formic acid in water; Eluent B = 1 % formic acid in methanol; time program = 0 min, 70 % A/30 % B; 9.0 min, 70 % A/30 % B; 9.1 min, 0 % A/100 % B; 11 min, 0 % A/100 % B; 11.1 min, 70 % A/30 % B; 15 min, 70 % A/30 % B. LC/MS/MS detection and quantification of 5MT/[$^{13}C_5$]-5MT and FA/[$^{13}C_5$]-FA was conducted in multiple reaction monitoring (MRM) mode. MRM transitions for each analyte were individually optimized for protonated analyte molecules $[M + H]^+$ and stable, protonated fragments. MRM mass transitions = *m/z* 460 → *m/z* 313 for 5MT, *m/z* 465 → *m/z* 313 for [$^{13}C_5$]-5MT, *m/z* 442 → *m/z* 295 for FA and *m/z* 445 → *m/z* 295 for [$^{13}C_5$]–FA. Analyte concentrations were calculated by linear interpolation from calibration curves constructed independently for each set of samples.

**Folate and Vitamin $B_{12}$ Measurements at CDC:** For the measurements of folates (5MT, FA, and 5FT) at CDC using LC/MS/MS [5], the target analytes were quantitatively isolated from 275 µL of the SRM 1955 serum using a phenyl solid-phase extraction cartridge, then detected and quantified in stabilized serum extracts by positive ion electrospray ionization LC/MS/MS using an isocratic mobile phase of acetic acid in organic solvent on a C–8 analytical column. $^{13}$C–labeled folates were used as internal standards. For the CDC microbiological assay for total folates [11], using a 96-well plate microtiter method, diluted SRM serum was added to an assay medium containing all of the nutrients except folic acid necessary for growth of *Lactobacillus casei* (*L. casei*, NCIB 10463). The assay medium was then inoculated with *L. casei* and the microtiter plate was incubated for 42 h at 37° C. Because the growth of *L. casei* is proportional to the amount of total folate present in the serum sample, the folate concentration was quantified by measuring the turbidity of the inoculated assay medium at 590 nm in a micro plate reader. For the CDC radioassay for total folates and vitamin $B_{12}$, a radio–protein binding assay was used. The SRM serum was combined with the tracers $^{125}$I-folate and $^{57}$Co-vitamin $B_{12}$. The mixture was boiled to inactivate endogenous folate-binding proteins and to convert the various forms of vitamin $B_{12}$ to cyanocobalamin. After cooling the mixture was combined with immobilized affinity-purified porcine intrinsic factor and folate-binding proteins, incubated for 1 h at room temperature, and finally centrifuged and decanted. The endogenous and labeled folate and $B_{12}$ compete for the limited number of binding sites on the basis of their relative concentrations and were concentrated in the bottom of the tube in the form of a pellet. The unbound folate and vitamin $B_{12}$ in the supernatant were discarded. The radioactivity associated with the pellet was counted. Standard curves were prepared by using the pre-calibrated folate/ vitamin $B_{12}$ standards in a human serum albumin base. The concentration of the folate and vitamin $B_{12}$ in the SRM was calculated from the standard curve.

Table 1. Certified Concentrations[a] and Uncertainties for Total Homocysteine and 5-Methyltetrahydrofolic Acid in SRM 1955

Concentration Levels for Homocysteine

| | µmol/L | µg/mL |
|---|---|---|
| Level I | 3.98 ± 0.18 | 0.538 ± 0.024 |
| Level II | 8.85 ± 0.60 | 1.196 ± 0.082 |
| Level III | 17.7 ± 1.1 | 2.39 ± 0.15 |

Concentration Levels for 5-Methyltetrahydrofolic Acid

| | nmol/L | ng/mL |
|---|---|---|
| Level I | 4.26 ± 0.25 | 1.96 ± 0.12 |
| Level II | 9.73 ± 0.24 | 4.47 ± 0.11 |
| Level III | 37.1 ± 1.4 | 17.03 ± 0.64 |

[a] Each certified value is the weighted mean from multiple methods. A Bayesian approach [12] was used to combine the data from the multiple methods to determine the combined standard uncertainties, $u_c$. The expanded uncertainties, $U$, equal $k$ times $u_c$,with $k = 2$, and conform with ISO guidelines for expression of uncertainty [13].

Table 2. Reference Concentrations[a] and Uncertainties for Folic Acid in SRM 1955

| | nmol/L | ng/mL |
|---|---|---|
| Level I | 0.49 ± 0.17 | 0.215 ± 0.075 |
| Level II | 1.05 ± 0.16 | 0.463 ± 0.071 |
| Level III | 1.07 ± 0.24 | 0.47 ± 0.11 |

[a] Each reference value is the weighted mean from multiple methods. A Bayesian approach was used to combine the data from the multiple methods to determine the combined standard uncertainties, $u_c$. The expanded uncertainties, $U$, equal $k$ times $u_c$,with $k = 2$, and conform with ISO guidelines for expression of uncertainty [13].

Table 3. Information Values[a] for Total Folate, 5-Formyltetrahydrofolic Acid, Total Homocysteine[b], and Vitamin $B_{12}$ Based upon Method Specific Determinations

Level I

| Analyte | Method | Concentration[a] | Units |
|---|---|---|---|
| Total Folate | LC/MS/MS | 6.0 ± 0.2 | nmol/L |
| Total Folate | Microbiological | 5.6 ± 0.7 | nmol/L |
| Total Folate | Radioassay | 4.5 ± 0.2 | nmol/L |
| 5-FT | LC/MS/MS | 0.012 ± 0.001 | nmol/L |
| Homocysteine | FPIA | 4.2 ± 0.2 | µmol/L |
| Homocysteine | FD | 3.5 ± 0.2 | µmol/L |
| Vitamin $B_{12}$ | Radioassay | 0.16 ± 0.01 | nmol/L |

Level II

| Analyte | Method | Concentration | Units |
|---|---|---|---|
| Total Folate | LC/MS/MS | 13.1 ± 0.7 | nmol/L |
| Total Folate | Microbiological | 14 ± 2 | nmol/L |
| Total Folate | Radioassay | 10.0 ± 0.4 | nmol/L |
| 5-FT | LC/MS/MS | 0.023 ± 0.006 | nmol/L |
| Homocysteine | FPIA | 8.6 ± 0.2 | µmol/L |
| Homocysteine | FD | 8.2 ± 0.3 | µmol/L |
| Vitamin $B_{12}$ | Radioassay | 0.36 ± 0.03 | nmol/L |

Level III

| Analyte | Method | Concentration | Units |
|---|---|---|---|
| Total Folate | LC/MS/MS | 41 ± 1 | nmol/L |
| Total Folate | Microbiological | 44 ± 7 | nmol/L |
| Total Folate | Radioassay | 25 ± 2 | nmol/L |
| 5-FT | LC/MS/MS | 0.037 ± 0.008 | nmol/L |
| Homocysteine | FPIA | 16.7 ± 0.4 | µmol/L |
| Homocysteine | FD | 16.7 ± 0.3 | µmol/L |
| Vitamin $B_{12}$ | Radioassay | 0.35 ± 0.03 | nmol/L |

[a] Sufficient information was not available for a complete evaluation of uncertainty for the individual method results. The uncertainties are ± 2 times the standard deviation of the mean of the measurements.

[b] See Table 1 for the certified values for homocysteine.

REFERENCES


[1] Nelson, B.C.; Pfeiffer, C.M.; Sniegoski, L.T.; Satterfield, M.B.; *Development and Evaluation of an Isotope Dilution LC/MS Method for the Determination of Total Homocysteine in Human Plasma*; Anal. Chem., Vol. 75, pp. 774–784 (2003).

[2] Satterfield, M.B.; Sniegoski, L.T.; Welch, M.J.; Nelson, B.C.; Pfeiffer, C.M.; *Comparison of Isotope Dilution Mass Spectrometry Methods for the Determination of Total Homocysteine in Plasma and Serum*; Anal. Chem., Vol. 75, pp. 4631–4638 (2003).

[3] Nelson, B.C.; Satterfield, M.B.; Sniegoski, L.T.; Pfeiffer, C.M.; Welch, M.J.; *Simultaneous Quantification of Homocysteine and Folate in Human Serum or Plasma Using Liquid Chromatography/Tandem Mass Spectrometry*; Anal. Chem., Vol. 77, pp. 3586–3593 (2005).

[4] Nelson, B.C.; Pfeiffer, C.M.; Margolis, S.A.; Nelson, C.P.; *Solid Phase Extraction-Electrospray Ionization Mass Spectrometry for the Quantification of Folate in Human Plasma or Serum*; Anal. Biochem.**,** Vol. 325, pp. 41–51 (2004).

[5] Pfeiffer, C.M.; Fazili, Z.; McCoy, L.; Zhang, M.; Gunter, E.W.; *Determination of Folate Vitamers in Human Serum by Stable-Isotope-Dilution Tandem Mass Spectrometry and Comparison with Radioassay and Microbiologic Assay*; Clin. Chem.,Vol. 50, pp. 423–432 (2004).

[6] The list of approved reference materials and reference measurement procedures can be found at http://www.bipm.fr/en/committees/jc/jctlm/jctlm-db/.

[7] May, W.E.; Parris, R.M.; Beck, C.M.; Fassett, J.D.; Greenberg, R.R.; Guenther, F.R.; Kramer, G.W.; Wise, S.A.; Gills, T.E.; Colbert, J.C.; Gettings, R.J.; MacDonald, B.S.; *Definitions of Terms and Modes Used at NIST for Value-Assignment of Reference Materials for Chemical Measurements*; NIST Special Publication 260-136 (2000). To obtain a copy see: http://ts.nist.gov/ts/htdocs/230/232/SP_PUBLICATIONS/documents/sp260-136.pdf.

[8] *Biosafety in Microbiological and Biomedical Laboratories*; U.S. Department of Health and Human Services; U.S. Government Printing Office: Washington, DC (1988).

[9] Pfeiffer, C.M.; Huff, D.L.; Gunter, E.W.; *Rapid and Accurate HPLC Assay for Total Plasma Homocysteine and Cysteine in a Clinical Lab Setting*; Clin. Chem., Vol. 45, pp. 290–292 (1999).

[10] Pfeiffer, C.M.; Twite, D.; Shih, J.; Holets-McCormack, S.R.; Gunter, E.W.; *Method Comparison for Total Plasma Homocysteine Between the Abbott IMx Analyzer and an HPLC Assay with Internal Standardization*; Clin. Chem., Vol. 45, pp. 152–153 (1999).

[11] Molloy, A.M.; Scott, J.M.; *Microbiological Assay for Serum, Plasma, and Red Cell Folate Using Cryopreserved, Microtiter Plate Method*; Methods Enzymol, Vol. 281, pp. 43–45 (1997).

[12] Liu, H-K.; Zhang, N.F.; *Bayesian Approach to Combining Results from Multiple Methods*; Proceedings of the Section of Bayesian Statistical Science of American Statistical Society (2001).

[13] ISO; *Guide to the Expression of Uncertainty in Measurement*; ISBN 92–67–10188–9, lst ed.; International Organization for Standardization: Geneva, Switzerland (1993); see also Taylor, B.N.; Kuyatt, C.E.; *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*; NIST Technical Note 1297, U.S. Government Printing Office: Washington, DC (1994); available at http://physics.nist.gov/Pubs/.


*Users of this SRM should ensure that the certificate in their possession is current. This can be accomplished by contacting the SRM Program at: telephone (301) 975-6776; fax (301) 926-4751; e-mail srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.*